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Neuroprotective effect of methanol extract of *Capparis spinosa* L. fruits in an in-vitro experimental model of Parkinson's disease

Capparis spinosa L. meyvelerinin metanol ekstraktının Parkinson hastalığının in-vitro deneysel modelinde nöroprotektif etkisi

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ABSTRACT

Aim: Parkinson's disease (PD) is the second most widespread neurodegenerative disease. This study, it was aimed to investigate the effect of methanol extract obtained from *Capparis spinosa* L. fruits, which are known to have important bioactive components, on in-vitro experimental PD model.

Material and Method: After collecting *Capparis spinosa* L. fruits from Alanya/Antalya, methanol extract was prepared by drying and grinding. SH-SY5Y cells grown in flasks were transferred to 96 well plates and were incubated until 80% cell density was reached. Different doses of methanol extract were applied to the cells 30 minutes before the PD model was formed. For the PD model, SH-SY5Y cells were exposed to 200 µM 6-OHDA for 24 hours. MTT analysis was performed to assess the viability of SH-SY5Y cells at the end of the 24-hour period. TOS, TAC, and IL-17A levels in the cell medium were determined using the ELISA method. Expression of TNFα and α-synuclein was defined using the immunohistochemical method.

Results: Cell viability was found to be higher in all treatment groups than in the 6-OHDA group. Moderate levels of TNFα and α-synuclein positivity were observed in the 1500 µg/ml methanol extract group. It was determined that TOS and TAC levels change depending on the dose. It has been determined that the level of IL-17A decreases at low doses. Statistical significance was found between the groups.

Conclusion: When the findings were examined, it was determined that the methanol extract obtained from *Capparis spinosa* L. fruits reduced oxidative stress and IL-17A levels at low doses and provided a neuroprotective effect by increasing the antioxidant capacity.

Keywords: *Capparis spinosa* L., SH-SY5Y, Parkinson's disease, neuroprotective

ÖZ

Amaç: Parkinson hastalığı, en sık görülen ikinci nörodejeneratif hastalıktır. Bu çalışmada, önemli biyoaktif bileşenlere sahip olduğu bilinen *Capparis spinosa* L. meyvelerinden elde edilen metanol ekstraktının in vitro deneysel Parkinson hastalığı modeline etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Alanya/Antalya'dan *Capparis spinosa* L. meyveleri toplandıktan sonra kurutulup öğütülerek metanol özütü hazırlandı. Flasklarda geliştirilen SH-SY5Y hücreleri, 96 oyuklu plakalara aktarıldı ve %80 hücre yoğunluğuna ulaşılana kadar inkübe edilmiştir. Parkinson hastalığı modeli oluşturulmadan 30 dakika önce hücrelere farklı dozlarda metanol özütü uygulanmıştır. Parkinson hastalığı modeli için SH-SY5Y hücreleri, 24 saat boyunca 200 uM 6-OHDA'ya maruz bırakılmıştır. 24 saatlik sürenin sonunda SH-SY5Y hücrelerinin canlılığını değerlendirmek için MTT analizi yapılmıştır. Hücre ortamındaki TOS, TAC ve IL-17A seviyeleri ELISA yöntemi kullanılarak belirlenmiştir. TNFα ve α-sinüklein ekspresyonu, immünohistokimyasal yöntem kullanılarak tespit edilmiştir.

Bulgular: Hücre canlılığının tüm tedavi gruplarında 6-OHDA grubuna göre daha yüksek olduğu bulundu. 1500 µg/ml metanol özütü grubunda orta düzeyde TNFα ve a-sinüklein pozitifliği gözlendi. TOS ve TAC düzeylerinin doza bağlı olarak değiştiği belirlendi. Düşük dozlarda IL-17A seviyesinin düştüğü bulundu. Gruplar arasında istatistiksel anlamlılık saptandı.

Sonuç: Bulgular incelendiğinde *Capparis spinosa* L. meyvelerinden elde edilen metanol ekstraktının düşük dozlarda oksidatif stres ve IL-17A düzeylerini azalttığı ve antioksidan kapasiteyi artırarak nöroprotektif etki sağladığı belirlendi.

Anahtar Kelimeler: *Capparis spinosa* L., SH-SY5Y, Parkinson hastalığı, nöroprotektif

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INTRODUCTION

PD is a neurodegenerative, progressive disease. PD, whose incidence increases with the aging population, has been predicted to affect approximately thirteen million people by 2040 (1). The increasing prevalence of PD has caused some health professionals to define it as a non-communicable pandemic disease like diabetes. This increase has been related to decreased smoking rates, increased exposure to environmental waste, and diet, in addition to the aging population (2,3).

The substantia nigra pars compacta (SNc) are one of the nuclei forming the basal ganglia. Progressive neurological degeneration in this region is the most important pathological sign of PD. Neurons located in the SNc are involved in the transmission of dopamine to other basal ganglia nuclei and the striatum. Degeneration of neurons in the SNc leads to dysfunction of neuronal circuits, including the basal ganglia and motor cortical areas including. This causes movement abnormalities that affect the individual's standard of living and are the main symptoms of PD. Motor dysfunctions and non-motor symptoms occur clinically in idiopathic PD. Motor dysfunctions; stiffness, tremors, loss of spontaneous movement, and impaired balance. Non-motor symptoms appear as dementia, sleep, mood, and personality changes (4).

Intracellular accumulation of misfolded α-synuclein protein in the striatum of PD patients is a histopathological distinctive finding. Among the mechanisms causing this pathophysiology, attention was drawn mostly to oxidative stress, mitochondrial dysfunction, apoptosis, and neuroinflammation. It is also known that the expression levels of proinflammatory cytokines such as IL-1β, IL-6, IL-17A, and TNFα increase in relation to neuroinflammation in these patients (4,5). In recent clinical studies, it has been reported that vitamins, β-carotene, caffeine, omega-3, omega-6, and Mediterranean diet supplements slow the progression of Parkinson's Disease and provide cognitive and behavioral improvement (6-8).

Cappari spinosa L. is a thorny perennial shrub belonging to the Capparidaceae. Widespread in the Mediterranean, it shows a wide distribution including Europe, Africa, Madagascar, and Asia. It is known that different parts of the plant have been used in the treatment of skin diseases, cough, asthma, headache, diabetes, ulcer, and sciatica since ancient times (9). During the excavations in Syria (Tell es-Sweyhat, Syria), the seeds of *Capparis spinosa* L. dated to 2400-1400 were discovered in a sarcophagus. It was concluded that these seeds were used for medicinal purposes by the researchers due to the presence of *Cannabis sativa* plant residues (10). The flower buds of the plant are utilized as a spice, while the fruits are consumed after fermenting (11). Various have been determined components of *Capparis spinosa* L. including alkaloids, furan, flavonoids, and pyrrole derivatives, phenolic acids, tetraterpenes, sterols, capparisine A, capparisine B, capparisine C, glucocapparin, isoginkgetin, ginkgetin, protocatechuic acid (11,12). It has been reported that methanol extracts of ripe fruits collected from Turkey contain cappariloside A, cappariloside B, gentisic acid, sinapinic acid, and benzoic acid (13,14). In clinical and experimental studies, it has been proven that extracts obtained from *Capparis spinosa* L. fruits have anti-viral, anti-diabetic, hypolipidemic, hepatoprotective, anti-hypertensive, and anti-inflammatory effects (15-19).

In this study, methanol extract obtained from *Capparis spinosa* L. fruits was investigated for the first time in experimental Parkinson's disease in-vitro and the neuroprotective effect of the extract was presented. Expression of α-synuclein, which has a specifically important place in the diagnosis of PD, was evaluated immunohistochemically. While oxidative stress level was determined by TAC and TOS biochemical analysis, neuroinflammation level was investigated using TNFα and IL-17A immunohistochemical and biochemical analyzes.

MATERIAL AND METHOD

This study was carried out using immortal cell lines. Ethics committee approval was not obtained because human or animal subjects were not used in the study. However, all procedures were carried out in accordance with ethical rules and the principles.

Capparis spinosa L. Extraction

All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki. *Capparis spinosa* L. (CS) fruits were collected from their natural environment in Alanya/Antalya in August. Dried and ground fruits (20 g) of CS were extracted with 300 ml of methanol utilizing a Soxhlet extractor (ISOPAD, Heidelberg, Germany) for 6 hours at a temperature below boiling point (<64°C). Extract filtered out Whatman fiber paper no 1 and then concentrated under vacuum at 40°C using an evaporator (Buchi Labortechnic AG, Flawil, Switzerland) and the extract, determined to have a yield of 10.5%, was stored at +4°C in the dark until use (20).

Cell Culture and MTT Test

DMEM/F12 medium containing 10% FBS and 1% antibiotic/antimitotic solution was prepared for cell culture. An amount of the prepared medium was taken and transferred to flasks together with the SH-SY5Y

cell line. The flask was incubated at 5% CO2 and 37°C. SH-SY5Y cells grown in flasks were then transferred to 96 well plates and incubated until 80% cell density was reached. Different doses of CS extract (1000-3000 µg/ml) were applied to the cells 30 minutes before the PD model was created. For the PD model, SH-SY5Y cells were exposed to 200 µM 6-OHDA for 24 hours. 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) (SigmaAldrich) analysis was performed to assess the viability of SH-SY5Y cells at the end of the 24-hour period. After applying 20 µL of MTT solution to all wells, it was incubated at 37 °C for 4 hours. For spectrophotometric examination of the formed formazan crystals, 150 µl of DMSO was added to the wells whose supernatants were removed. Completely dissolved formazan crystals were measured at 570 nm with the aid of a spectrophotometer. Statistical analyses were performed using one-way analysis of variance (ANOVA) with post hoc Tukey's test (IBM SPSS 22.0) ($p<0.05$, $p<0.001$) (21,22).

Immunohistochemical Analysis

Based on the results from the MTT assay, the in vitro model of PD and the administration of the extract at a dose range of 1500-2500 µg/ml were repeated in 24-well plates for immunohistochemistry staining. After 24 hours of exposure, SH-SY5Y cells were fixed with methanol for 5 minutes at -20°C and washed with PBS (Phosphate Buffered Saline). Then, PBS containing 0.1% Triton X-100 was added and incubated at 22°C for 15 minutes. After washing, it was incubated with PBS containing 2% Bovine Serum Albumin for 1 hour at room temperature. After rewashing, it was incubated overnight at +4°C with monoclonal anti-α-synuclein (Santa cruz, sc-69977), and monoclonal anti-TNFα (Santa cruz, sc-52746) primary antibodies at a dilution ratio of 1/300. Cells washed away with PBS were incubated with goat anti-mouse (FITC) secondary antibody (Jackson ImmunoResearch, no: 115-095-003) at a dilution of 1/50 for 1 hour at 22°C in the dark, consistent with the primary antibodies used. Lastly, DAPI (4',6-diamidino-2-phenylindole) was instilled into the washed cells and investigated under a fluorescent microscope (Zeiss Axio). In the examination, the density of fluorescence positivity in the cells was evaluated with the Fiji Image J program.

In the data analyzed with the SPSS 20.00 program, the difference between the groups was determined by the Kruskal Wallis test, and the group that created the difference was determined by the Mann Whitney U test ($p<0.05$) (23).

Biochemical Analysis

Oxidative stress parameters, total oxidant status (TOS), and total antioxidant capacity (TAC) in the medium of SH-SY5Y cells for which an in-vitro PD model was created were investigated (Rel Assay Diagnostics, Gaziantep, Turkey). The level of IL-17A (BT LAB, Zhejiang, China) in the medium related to neuro-inflammation was determined in accordance with the protocol recommended by the ELISA kit manufacturer. Biochemical analysis was performed with post hoc Tukey test (IBM SPSS 22.0) ($p <0.05$, $p<0.001$) and one-way analysis of variance (ANOVA) (23,24).

RESULTS

MTT Test

Compared with the 6-OHDA group, cell viability was found to be higher in all treatment groups. While the cell viability changed depending on the dose, the groups with the highest cell viability were determined as the groups treated with 1000-1500 µg/ml CS methanol extract. A statistically significant difference was defined in all treatment doses compared to the 6-OHDA group (**Figure 1**).

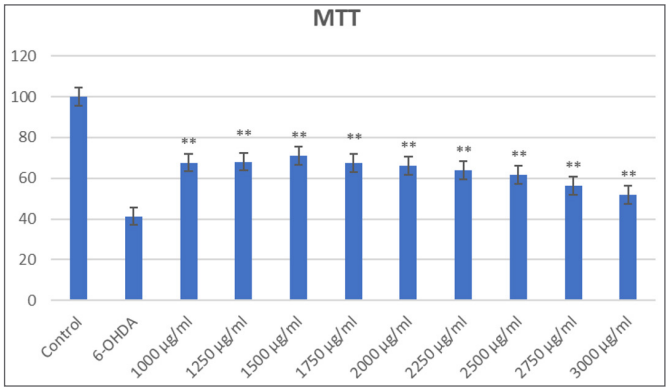

Figure 1. MTT analysis results. *p<0.05, **p<0.001

Immunohistochemical Results

A statistically significant difference was defined between the groups in immunofluorescent staining with α-synuclein and TNFα (**Table 1**, $p<0.001$). While mild fluorescence positivity was a sight in the control group, severe positivity was detected in the 6-OHDA group. In the application groups, moderate positivity was found at 1500 µg/ml, and severe positivity at 1750-2500 µg/ml (**Figure 2**).

Table 1. Statistical differences in α-synuclein and TNFα immunofluorescence staining

Groups	α-synuclein	TNFα
Control	28.42±1.92[a]	30.13±0.52[a]
6-OHDA	71.62±0.43[b]	73.41±1.38[b]
1500 µg/ml	54.07±0.25[c]	58.24±1.34[c]
1750 µg/ml	69.92±0.34[b]	74.04±1.82[b]
2000 µg/ml	70.71±0.22[b]	73.22±0.62[b]
2250 µg/ml	71.46±1.34[b]	72.49±0.50[b]
2500 µg/ml	68.16±1.69[b]	72.81±1.63[b]

a,b,c indicate the difference between groups, p<0.001.

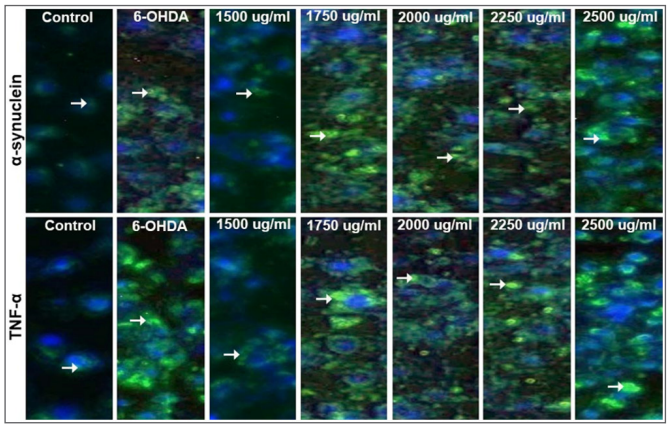

Figure 2. α-synuclein and TNFα expression levels

Control group-Mild, 6-OHDA group-Severe, 1500 µg/ml group-Moderate, 1750 µg/ml, 2000 µg/ml, 2250 µg/ml, and 2500 µg/ml groups-Severe positivity (arrows), IF.

Biochemical Analysis Results

It was determined that the oxidative level in the medium of the PD-modeled cells increased at high doses, while it decreased at low doses consistent with the MTT test. The antioxidant capacity was detected to be at the highest level at 1500 µg/ml and 1750 µg/ml doses. Besides, antioxidant levels were higher in all treatment groups than in the control and 6-OHDA groups, except for the 2750 µg/ml and 3000 µg/ml doses. Statistically, a significant difference was determined in all treatment groups (**Figure 3**, **Figure 4**). The group with the highest level of IL-17A, a proinflammatory cytokine, was found as 6-OHDA. It is remarkable that the IL-17A level increased with increasing doses in the treatment groups. A statistically significant difference was determined in all groups compared to the 6-OHDA group (**Figure 5**).

DISCUSSION

PD is an important neurodegenerative disease with an increasing incidence. In this study, the application of methanolic extract obtained from CS fruits in the dose range of 1000 µg/ml-2250 µg/ml increased cell viability in the PD model created using the SH-SY5Y cell line. It has also been determined that it has shown antioxidant and anti-inflammatory effects. In immunohistochemical analyzes, moderate α-synuclein and TNFα expression have been detected at a dose of 1500 µg/ml. When the results obtained were evaluated, it was determined that the methanol extract of CS fruits showed a dose-dependent neuroprotective effect in the in-vitro PD model.

Classified among medicinal plants, CS is used in the remedy of diverse illnesses due to the flavonoids, alkaloids, and phenolic acids it contains. Studies have shown that CS fruits contain p-Coumaric acid (5.53 ± 0.01 mg/100 g DW), Ferulic acid (0.73 ± 0.00 mg/100 g DW), Catechin (0.91 ± 0.02 mg/100 g DW), Epicatechin (1.14 ± 0.01 mg/100 g DW), Rutin (17.12 ± 0.00 mg/100 g DW), Kaempferol (3.44 ± 0.01 mg/100 g DW), and Quercetin

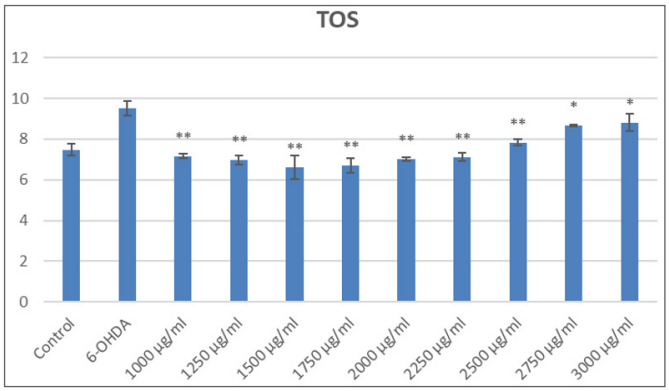

Figure 3. TOS analysis results

*p<0.05, **p<0.001

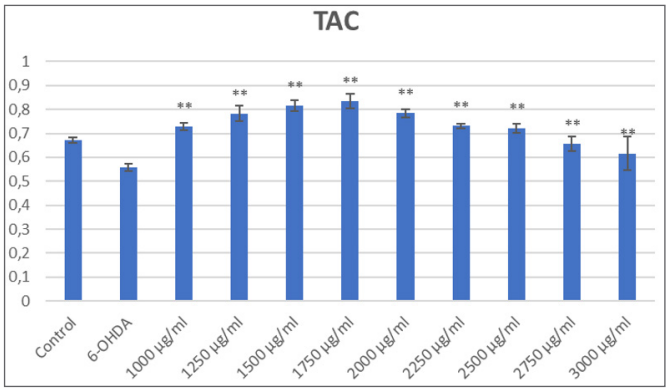

Figure 4. TAC analysis results

*p<0.05, **p<0.001

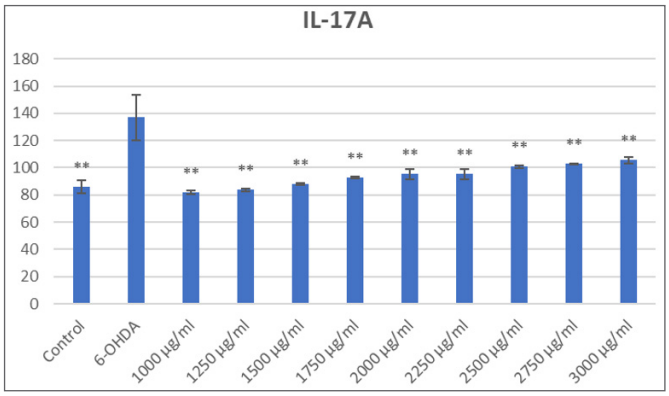

Figure 5. IL-17 analysis results

*p<0.05, **p<0.001

(0.29 ± 0.01 mg/100 g DW) (25). Zhu et al, who created an experimental ulcerative colitis model in mice, have been used water extracts of CS fruits in the treatment. They have reported that after seven days of treatment, the expression IL-6, IL-1β, and TNFα was suppressed, and improvement was achieved in mice by reducing oxidative stress (26). In another study, a streptozotocin-induced diabetes model was established in rats by Jalali et al. (27) and diabetic rats were treated with water extracts from CS fruits for 28 days. As a result of the analysis, they determined that it lowered blood sugar and lipid levels independently of insulin. In a randomized controlled trial, Vahid et al. (18) reported that CS oxymel treatment applied to patients diagnosed with type 2 diabetes did

not show any difference in HbA1c level compared to the placebo group, however, it prevented the progression of hyperglycemia. In terms of the components, it contains, CS has a strong antioxidant effect. It was determined that CS ethanol extract reduced Doxorubicin-induced apoptotic induction and cardiotoxicity in cardiomyoblast cells (28). It has been proven that hydro-ethanolic extract of CS aboveground parts significantly reduces IL-1β, NO, iNOS, PGE2, IL-6, TNF-α, and COX-2 levels in brain tissue and improves cognitive impairment associated with lipopolysaccharide-induced inflammation (29). They found that it increased learning and memory functions in mice treated with CS methanol extract in an Alzheimer's disease (AD) model induced by D-galactose injection in mice. It is known that the D-galactose model acts on oxidative stress. In this study, in which SOD, CAT and GPx activities increased, the improvement in AD model was associated with antioxidant mechanisms (30). 6-OHDA, which is frequently preferred in experimental PD models, is structurally similar to dopamine and norepinephrine. By inducing mitochondrial dysfunction, 6-OHDA increases oxidative stress, leading to neuronal degeneration (23). In our study, it was determined that in the in-vitro PD model created by 6-OHDA in SH-SY5Y cells, the total oxidant level decreased, and the antioxidant level increased in a dose-related manner in the treatment groups.

In a model of AD induced by injection of amyloid-beta peptide in rats, CS hydroalcoholic extracts have been shown to provide more effective up-regulation of the expression of genes encoding Gamma-secretase and Beta-secretase enzymes compared to the Rutin-treated group. In this study, the neuroprotective effect was associated with anti-inflammatory mechanisms as well as the antioxidant effect of the bioactive compounds contained in CS (31). The proinflammatory cytokines IL-17A and TNFα, which are known to increase in neurodegenerative disorders, were found to increase in our study in a dose-dependent manner. In addition, it was determined that α-synuclein expression, which is a distinctive biomarker in the diagnosis of PD, was moderate at 1500 μg/ml, and severe in 6-OHDA and high-dose treatment groups. This situation was thought to be related to the doses administered.

CONCLUSION

This study, the neuroprotective effect of methanol extract of *Capparis spinosa* L. fruits on in-vitro PD was investigated for the first time and it was proven that the extract provided a dose-dependent neuroprotective effect. These findings showed that the methanol extract of *Capparis spinosa* L. fruits with its antioxidant and anti-inflammatory properties may be a potential candidate for neuroprotection in PD. In-vivo experiments and detailed analysis are needed to understand the efficacy and mechanism.

ETHICAL DECLARATIONS

Ethics Committee Approval: This study was carried out using immortal cell lines. Ethics committee approval was not obtained because human or animal subjects were not used in the study.

Informed Consent: Since this study used an immortal cell line and was not a human study, written consent is not required.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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